

Characterization of Estrogenicity of Phytoestrogens in an Endometrial-derived Experimental Model

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Severe developmental and reproductive disorders in wild animals have been linked to high exposure to persistent environmental chemicals with hormonal activity. These adverse effects of environmental estrogens have raised considerable concern and have received increasing attention. Although numerous chemicals with the capacity to interfere with the estrogen receptor (ER) have been identified, information on their molecular mechanism of action and their relative potency is rather limited. For the endometrium, the lack of information is due to the lack of a suitable experimental model. We investigated the functions of phytoestrogens in an endometrial-derived model, RUCA-I rat endometrial adenocarcinoma cells. The cells were cultured on a reconstituted basement membrane to preserve their functional differentiation and estrogen responsiveness. We assessed the relative binding affinity to the estrogen receptor of the selected phytoestrogens coumestrol, genistein, daidzein, and the putative phytoestrogen mangostin compared to estradiol by a competitive Scatchard analysis. The following affinity ranking was measured: 17β -estradiol \gg coumestrol $>$ genistein $>$ daidzein \gg mangostin. In addition, we investigated the capacity of these compounds to promote the increased production of complement C3, a well-known estradiol-regulated protein of the rat endometrium. All substances tested increased the production of complement C3, although different concentrations were necessary to achieve equivalent levels of induction compared to estradiol. Mechanistically we were able to demonstrate that the increase of complement C3 production was mediated by primarily increasing its steady-state mRNA level. These findings indicate that RUCA-I cells represent a sensitive model system to elucidate relative potencies and functions of environmental estrogens in an endometrium-derived model. **Key words:** binding affinity, complement C3, endometrium, estrogen receptor, extracellular matrix, phytoestrogens. *Environ Health Perspect* 106:581-586 (1998). [Online 12 August 1998]
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Accumulated evidence of the past 30 years shows that humans and animals are exposed to a variety of persistent environmental xenoestrogens. These xenoestrogens have the potential to affect the endocrine system and cause developmental, endocrine, and oncological disorders. Potential effects of environmental estrogens on human health are intensely debated with regard to declining sperm quantity and quality in men (1-3) and as a cause of increased rates of breast cancer (4,5). Finally, there is clear evidence that offspring exposed *in utero* to diethylstilbestrol are at an increased risk for the development of reproductive tract abnormalities and rare vaginal clear-cell adenocarcinomas (6,7). The possible pathways for xenoestrogens to interfere with the endocrine system are manifold, and involve inhibition of biosynthesis of steroids and agonistic or antagonistic effects associated with binding to the estrogen receptor (ER) as well as modifying bound-to-free estradiol ratios. Endocrine disruptors can also bind to a nonsteroidal receptor like the aryl hydrocarbon receptor and exert estrogenic and antiestrogenic properties. Others act as estrogens apparently without mediation of the receptor.

Exposure to estrogenic industrial chemicals may cause adverse endocrine effects, whereas exposure to phytoestrogens such as

genistein, daidzein, and coumestrol may induce beneficial effects. Many clinical and epidemiologic studies describe a positive correlation between a phytoestrogen-rich diet and decreased risk for the development of hormone-dependent cancers such as prostate and breast cancer (8). Most phytoestrogens bind to the estrogen receptor with a lower affinity than 17β -estradiol (E_2) (9) and are weakly estrogenic (10). The competition of these weak estrogens with E_2 for nuclear binding might explain antiestrogenic features of these substances *in vivo* mainly observed regarding infertility and uterine wet weight (11,12). But to date, no conclusive evidence exists that phytoestrogens act as antiestrogens. The molecular basis of the protective effects of phytoestrogens remains unclear. To increase our knowledge about environmental estrogens, additional tissue-specific model systems are required to aid in their identification, assess their potency, and elucidate their mechanisms of action. Thus, it is important to have tissue-specific model systems representing various endocrine-responsive targets (e.g., breast as well as endometrium).

To obtain a more comprehensive picture of the effects of phytoestrogens in endometrial adenocarcinoma cells, we used

multiparameter analyses to assess the relative potency of the phytoestrogens coumestrol, genistein, daidzein, and mangostin. The binding affinity of the tested components to the ER and effects on formation and secretion of complement C3 served as parameters. The level of hormonal regulation of gene expression was assessed by Northern blot analyses. For all experiments we used the rat endometrial adenocarcinoma cell line RUCA-I, recently established in our laboratory. This cell line contains a relative high ER level (13). Cultivation of these cells on a reconstituted basement membrane in the presence of serum-free defined medium induced hormone responsiveness as well as morphological differentiation (14,15). The study presented here demonstrates that this cell line can be a valuable model system to investigate the effects of phytoestrogens on endometrial adenocarcinoma cells.

Materials and Methods

Hormones. The following control substances were used for the treatment of RUCA-I cells: E_2 (10^{-7} – 10^{-8} M, 99% purity; Sigma, Deisenhofen, Germany) and the pure antagonist ICI 164384 (5×10^{-7} – 5×10^{-8} M, research grade purity; kindly provided by A.E. Wakeling, Zeneca Chemicals, Macclesfield, UK) as well as tamoxifen (10^{-6} M, >99% purity; Sigma), which has partial agonistic and/or antagonistic properties. The following phytoestrogens were used as test substances: coumestrol (10^{-6} – 10^{-8} M, 95% purity; Fluka, Deisenhofen, Germany) genistein (10^{-6} – 10^{-9} M, 98% purity; Sigma), daidzein (10^{-6} – 10^{-9} M, purity determined by thin layer chromatography; Roth, Karlsruhe, Germany), and the putative phytoestrogen mangostin (10^{-5} – 10^{-8} M, 90% purity; a generous gift from L.A. Sopasorya, Bandung, Indonesia). The structure of control substances and investigated phytoestrogens is shown in Figure 1.

Cell culture. RUCA-I cells were precultured for 2 days on plastic in Dulbecco's modified Eagle's medium (DMEM)/F12

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medium containing 5% dextran-coated-charcoal-stripped (DCC) serum. After harvesting, 300,000 RUCA-I cells were seeded on top of 230 μ l extracellular matrix (Harbour Matrix, TEBU, Frankfurt, Germany) per well of a 24-well dish and cultured in the presence of 2 ml of a previously described serum-free defined medium (SFDM) (15) for 80 hr, the first 24 hr without additional hormonal treatment. Medium was changed daily, and hormonal treatment was repeated. To investigate potential estrogenic effects of compounds released from plastic culture dishes, RUCA-I cells were also cultured on top of an extracellular matrix plated on glass culture dishes.

Hormonal treatment. Following 24 hr culture on top of an extracellular matrix, RUCA-I cells were treated with the estrogenic and antiestrogenic substances described above for 56 hr. Estrogenic and antiestrogenic substances were added from stock solutions in ethanol at a volume of 0.1% of total culture medium. Controls received ethanol only.

Metabolic labeling of secretory proteins. After 24–48 hr of hormonal treatment, secretory proteins were labeled metabolically with 35 S-methionine (DuPont, Brussels, Belgium). For this purpose cells were cultured for another 16 hr in 150 μ l medium per well, whose methionine content was reduced by 90% and substituted with 200 μ Ci/ml 35 S-methionine. Thereafter supernatants containing *de novo* synthesized metabolically labeled secretory proteins were collected and centrifuged (3 min at 3000g). We used 15 μ l of the supernatant to determine the incorporation rate of the radioactive amino acid into trichloroacetic acid-precipitable material; the remainder was used for electrophoresis in 6% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Electrophoresis. Metabolically labeled proteins were separated according to standard protocols using a discontinuous system (16), nonreducing conditions, and 6% SDS-polyacrylamide gels. The same amount of radioactive counts of each experimental condition was loaded per lane onto the gel. After electrophoresis, gels were fixed in 10% acetic acid, 30% methanol, incubated in En³hance (DuPont), and dried for 1 hr at 80°C under vacuum. Bands were visualized by autoradiography. For semi-quantitative densitometric analysis, autoradiographs were scanned with the JX-330 scanner and the JX-3F6 scanning unit. The size and intensity of bands were analyzed with the Bio Image Intelligent Quantifier (IQ) program, version 2.1, installed on a Macintosh computer.

Ligand-binding assay. The ligand-binding affinity of the estrogenic and antiestrogenic

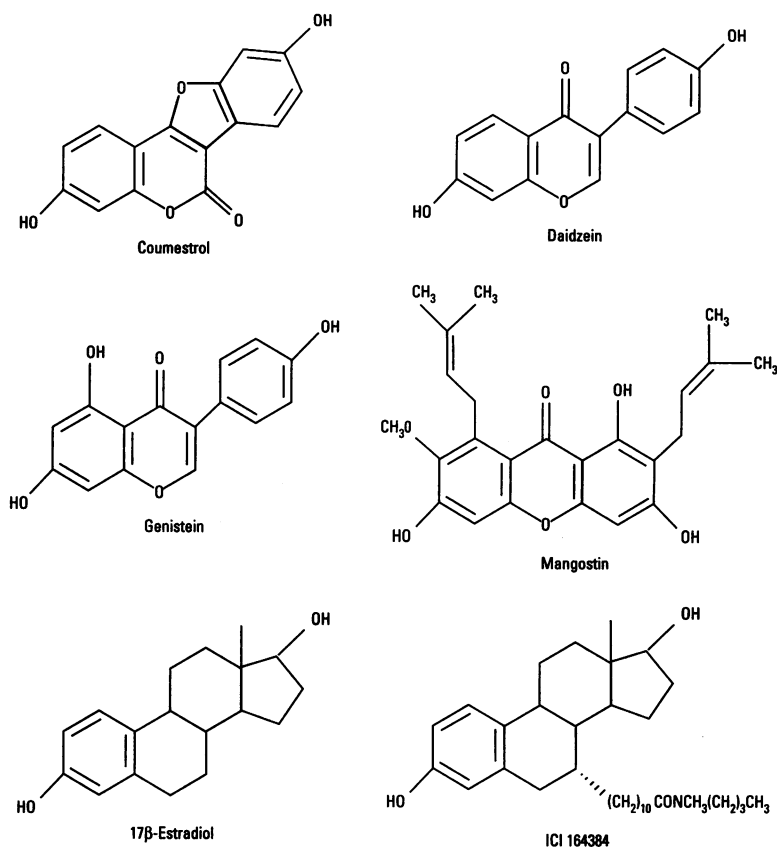


Figure 1. Molecular structures of 17 β -estradiol, the pure antagonist ICI 164384, and phytoestrogens coumestrol, genistein, daidzein, and the putative estrogen mangostin.

Table 1. Relative binding affinities of coumestrol, genistein, daidzein, and mangostin compared to 17 β -estradiol

Evaluation method	Coumestrol (%)	Genistein (%)	Daidzein (%)	Mangostin (%)
y-Axis	2.15 \pm 1	0.82 \pm 0.48	0.015 \pm 0.011	0.00075 \pm 0.00017
Slope	1.88 \pm 0.84	0.92 \pm 0.4	0.015 \pm 0.01	0.00078 \pm 0.00018

Binding affinities were evaluated by using the slope or the intersection of y-axis of the Scatchard analysis. Both evaluation methods gave similar results.

substances described above was assessed by a competitive Scatchard analysis as described previously (17,18) using the cytosol of RUCA-I cells. For this purpose RUCA-I cells were cultured on plastic to near confluency in the presence of DMEM/F12 medium containing 5% DCC serum, then trypsinized, pelleted by centrifugation (800g, 4°C), and washed twice with phosphate-buffered saline. The pellet was resuspended in an extraction buffer (19) (0.01 M Na₂MoO₄, 0.01 M NaH₂PO₄, 0.0015 M Na-EDTA, 10% glycerol, 0.01 M monothio-glycerol), and the cells were lysed by three freeze-thaw cycles in liquid nitrogen. The lysed cells were centrifuged for 45 min (105,000g, 4°C) to yield the supernatant (=cytosol). Total binding was assessed by incubation of aliquots (100 μ l) of cytosol with eight different concentrations of [3 H] E₂ (Amersham, Braunschweig, Germany) ranging from 0.05 to 1 nM for 18 hr at 4°C. To compare the binding affinity of

tested compounds to that of estradiol 1–10 pM, unlabeled estradiol was additionally added and used to generate a reference. For comparative purposes the ideal concentration of the competitor (either estradiol as a reference or phytoestrogens as test compounds) should be titrated to a concentration that competes for 30–70% of the binding activity measured for [3 H]-E₂ alone. The binding affinity of the unlabeled competitors was determined as described above for unlabeled estradiol except that coumestrol, daidzein, genistein, or mangostin were used at concentrations ranging from 10⁻⁵ to 10⁻⁹ M to compete for binding of [3 H]-E₂. For the estimation of nonspecific binding, 200 nM of unlabeled estradiol was added to the incubation mixture. After incubation, free steroids were absorbed from samples by treatment with charcoal dextran for 10 min at 4°C. Specific binding was then calculated from total binding minus nonspecific binding values.

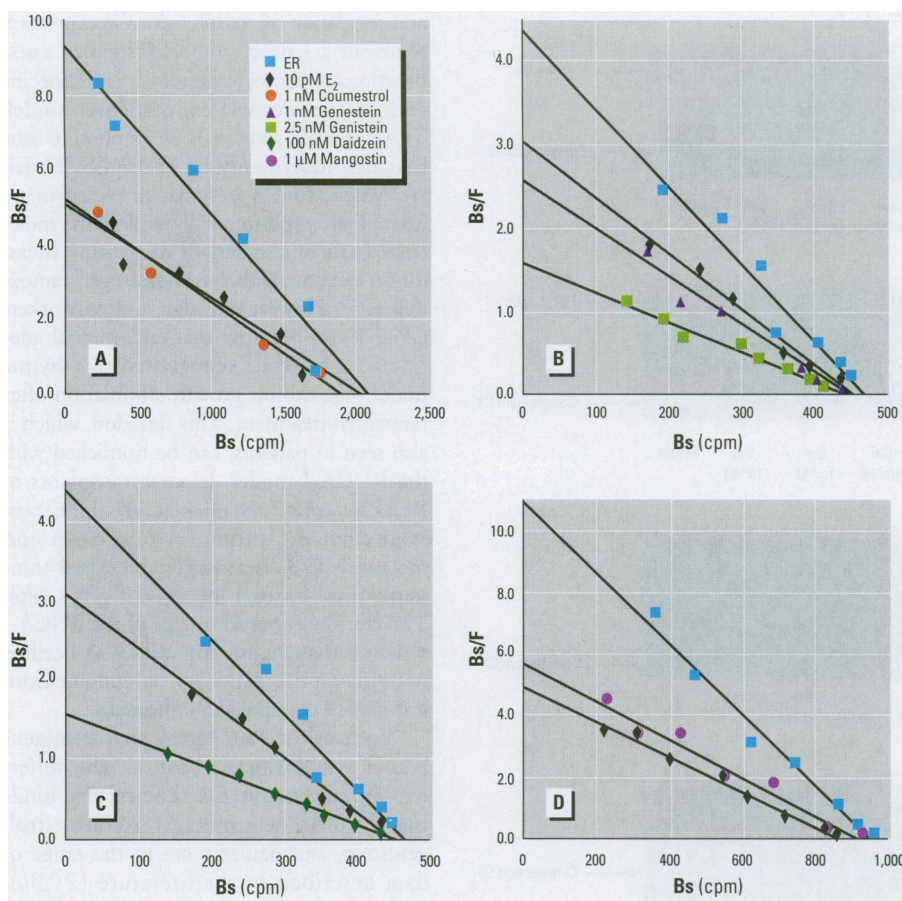


Figure 2. Relative binding affinities of the phytoestrogens (A) coumestrol, (B) genistein, (C) daidzein, and (D) mangostin evaluated by competitive binding assay according to Scatchard. Abbreviations: ER, estrogen receptor; E_2 , 17 β -estradiol; cpm, counts per minute; Bs, specific bound; Bs/F, ratio of specific bound to free hormone.

Results

Binding affinity of selected phytoestrogens.

The relative binding affinity of the phytoestrogens coumestrol, genistein, daidzein, and the putative phytoestrogen mangostin to the ER was assessed by a competitive ligand-binding assay according to Scatchard (17,18). Using the cytosolic preparation of rat endometrial adenocarcinoma cells, the following affinity ranking to the ER was evaluated: $E_2 \gg \text{coumestrol} > \text{genistein} > \text{daidzein} \gg \text{mangostin}$. The binding affinities of the phytoestrogens compared to E_2 ranged from approximately 2% for coumestrol to 0.00075% for mangostin (Table 1). The evaluation of binding affinities by either slopes of the linearized Scatchard curves or their intersections with y-axis lead to comparable results. In Figure 2 representative examples from competitive Scatchard binding assays are shown. From Scatchard analyses, general binding characteristics of the ER, such as the maximum of available binding sites and dissociation constant, can be determined. The mean ER content in RUCA-I cells originating from passage 30 for these series of experiments

was calculated to be 65.5 ± 15.5 fmol/mg cytosolic protein, and the high affinity is demonstrated by the mean dissociation constant of estradiol to the ER of $1.15 \pm 0.36 \times 10^{-11}$ mol/L.

Effects of phytoestrogens on formation and secretion of complement C3. The ER-positive RUCA-I cells respond to hormonal treatment if cultured on a reconstituted basement membrane. In this cell culture model the formation of complement C3 is induced by E_2 and is repressed by the pure antiestrogen ICI 164384 (15). Changes in *de novo* synthesis of secreted proteins after treatment with hormones, hormonelike substances, and antihormones were assessed by metabolic labeling focused on the analysis of complement C3. This protein is the major estradiol-regulated secretory protein of the normal rat uterus *in vivo* (20,21). The metabolic labeling experiments revealed that the phytoestrogens coumestrol, genistein, and daidzein increased *de novo* synthesis of secreted complement C3. Mangostin did not significantly alter the formation of complement C3 (Figs. 3 and 4). In our model system tamoxifen acts as an estradiol agonist at a concentration

of 10^{-6} M, whereas ICI 164384 is antagonistic and represses production of complement C3 even below control levels (Fig. 4). Semiquantitative densitometric analysis (Fig. 4) revealed that the increase in production of complement C3 protein by E_2 (10^{-8} M) was about twofold, and the induction at a higher concentration of E_2 (10^{-7} M) was slightly smaller. The phytoestrogens coumestrol, genistein, and daidzein had to be administered at an approximately 100 times higher concentration compared to E_2 to induce the same estrogenic effect (Fig. 4). Additionally, this study clearly showed that the degree of induction of complement C3 production mediated by phytoestrogens coumestrol, genistein, and daidzein did not correlate to the level that would have been predicted from their relative binding affinity to the ER. The three compounds had strikingly different binding affinities to the ER (Table 1), but all three (at a concentration of 10^{-6} M) increased the formation of complement C3 almost equally to about twofold. Providing further evidence that induction of complement C3 by the phytoestrogens, as exemplified by coumestrol, is nevertheless an ER-mediated effect, increasing concentrations of ICI 164384 were simultaneously added to coumestrol-treated cells (Fig. 5). The administration of ICI 164384 resulted in a reduction of coumestrol-induced formation of complement C3 in a dose-dependent manner. The highest concentration of ICI 164384 (5×10^{-7} M) resulted in a more than twofold inhibition. Culture of RUCA-I cells on top of extracellular matrix in glass culture dishes resulted in a reduction of control levels of complement C3 (data not shown), but this effect varied strongly between single experiments.

Regulation of Complement C3 Steady-state mRNA Level by Phytoestrogens. To investigate the level of regulation of induction of complement C3, Northern blot analyses were performed. As shown in Figure 6, the steady-state mRNA level of complement C3 is clearly increased by E_2 and by the phytoestrogens daidzein and genistein. The putative phytoestrogen mangostin leaves the level of complement C3 mRNA unchanged.

Discussion

The rat endometrial adenocarcinoma cell line RUCA-I represents a valuable model system for the assessment of ER-mediated estrogenic potency of environmental estrogens. These cells contain a relatively high amount of ER (13) compared to other endometrial cell lines (22) and respond to E_2 treatment with increased proliferation and increase of estrogen-dependent complement C3 production (14,15,23). RUCA-I cells

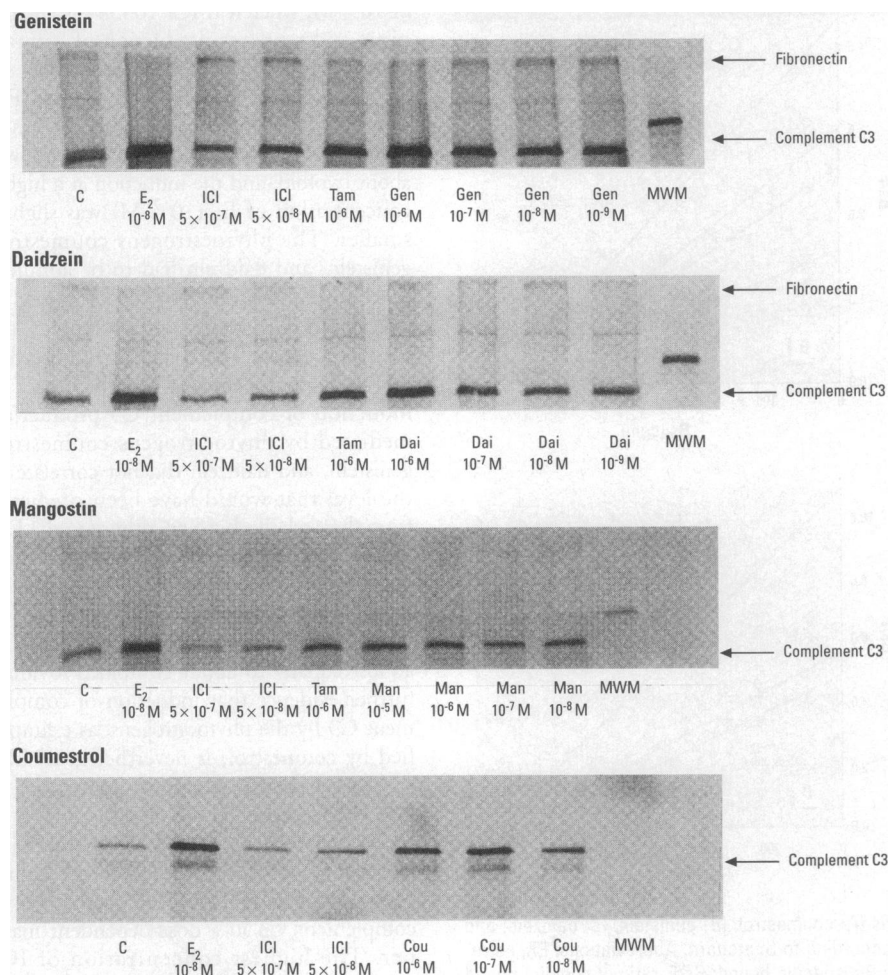


Figure 3. Effects of estrogenic substances on *de novo* synthesis of complement C3. C, Control; E₂, 17 β -estradiol; ICI, ICI 164384; Tam, tamoxifen; Gen, genistein; Dai, daidzein; Man, mangostin; Cou, coumestrol; MWM, molecular weight marker.

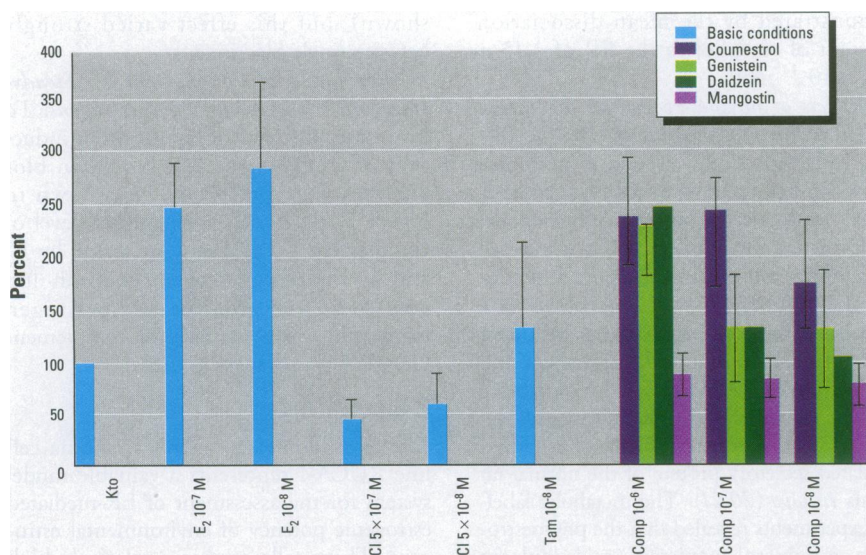


Figure 4. Effects of phytoestrogens on production of complement C3. Semiquantitative evaluation by densitometry of autoradiographs. Abbreviations: E₂, 17 β -estradiol; ICI, ICI 164384; Tam, tamoxifen; Comp, tested substance. Number of experiments: $n = 3$ for 10^{-3} M E₂, $n = 7$ for 10^{-8} M E₂, $n = 8$ for 5×10^{-7} M ICI 164384, $n = 4$ for 5×10^{-8} M ICI 164384, $n = 8$ for tamoxifen, $n = 3$ for genistein except 10^{-5} M ($n = 2$), $n = 2$ for daidzein, and $n = 3$ for mangostin except 10^{-5} M ($n = 2$). Error bars indicate standard deviations.

and regulation of complement C3 expression represent a sensitive model system to assess functions of xenoestrogens in a tissue-specific endometrial-derived experimental model. Tissue-specific analysis is an important issue that can most easily be exemplified with tamoxifen. Jordan (24) has provided many examples to experimentally explore the molecular basis of the benefit of treating breast cancer patients with the antiestrogen tamoxifen (25). However, Jordan and co-workers (26,27) also reported that endometrial adenocarcinoma cells xenografted to athymic nude mice exhibit growth stimulation after tamoxifen treatment. This situation, which is also seen in patients, can be mimicked with the RUCA-I-model. In xenotransplants of RUCA-I cells to syngeneic DA-Han rats, tamoxifen is a promotor of growth and metastasis (23) and triggers increased transcription of estrogen-dependent genes (26). Therefore, another advantage of the RUCA-I system is that any finding in RUCA-I cells is not only representative for rat endometrium but may be extrapolated to humans.

Each of the four tested phytoestrogens bound with distinct and considerably different affinities to the ER. The relative binding affinities determined for coumestrol, genistein, and daidzein are in the range of data described in the literature (29,30), which is important because the ER of RUCA-I cells exhibit an unusually low K_D between 1×10^{-11} and 1×10^{-12} M. These three phytoestrogens are able to increase the formation of complement C3. The difference in binding affinity to the ER among the phytoestrogens, which are capable of inducing gene expression, is almost two orders of magnitude and spans from coumestrol, the phytoestrogen with the highest binding affinity, to daidzein, the phytoestrogen with the lowest binding affinity. Despite different binding affinities, genistein and daidzein at a concentration of 10^{-6} M, and coumestrol at a concentration of 10^{-7} M increase formation of complement C3 more than 200%. Consequently, the degree of increase of protein formation is not strongly correlated with their binding affinity to the ER. This means that investigations using only binding affinity to assess estrogenic potency of environmental estrogens can yield misleading conclusions.

The apparent disagreement between the binding affinity of genistein and daidzein, which is around 50-fold, and the almost equipotent induction of molecular endpoints by these substances is surprising and might be caused by several nonestrogenic features of genistein. Genistein is known to be an inhibitor of phosphotyrosine kinases (31) and topoisomerases I and II (32). These two features of genistein interfere severely with

essential functions of cells. The reduction of phosphorylation by genistein can shut down signal transduction cascades, resulting in reduced proliferation. It is also known that genistein inhibits the tyrosine-specific kinase activity of the epidermal growth factor (EGF) receptor *in vitro* (31), which in turn is known to trigger ligand-independent activity of the ER (33). The mechanism of cross-talk between EGF and ER has not been elucidated yet, but it is known that it is not mediated by protein kinase C or protein kinase A (34). Genistein binds to the ER but potentially interferes additionally with ligand-independent estrogenic pathways.

Another cause for the lack of correlation between relative binding affinity and biological potency on gene expression may be the feature of some phytoestrogens to exhibit antiestrogenic properties in addition to their well-known agonistic effects (35). In our assays we failed to inhibit the effect of estradiol with phytoestrogens (data not shown). However, if we use fibronectin as a marker (Fig. 3), which in RUCA-I cells is an anti-estrogen-induced protein (14), and therefore represents antiestrogenicity, we find a relatively strong induction by genistein and a weak induction by daidzein. These diverging results are difficult to explain, but it has been suggested that the effect of an exogenous weak estrogen may act agonistically and/or antagonistically depending on endogenous steroid levels (36,37). Again the situation in RUCA-I cells appears different, as levels of complement C3 (agonistic response) rise at the same time as those of fibronectin (antagonistic response) after genistein treatment. The regulatory features of phytoestrogens on complement C3 expression resemble those of sex hormone-binding globulin production by endogenous steroids (38) and isoflavonoids (39) in HepG2 liver cells. In this system there is also no correlation between relative binding affinities and biological response. However, there is also one major dissimilarity between the two systems. Complement C3 expression is clearly regulated by a transcriptional mechanism, whereas the regulation of sex hormone-binding globulin appears to be post-transcriptionally regulated (39).

Recent publications show that coumestrol exhibits comparatively strong estrogenic effects (40), such as increasing uterine wet weight. On the other hand, coumestrol can cause hypermethylation of the *H-ras* proto-oncogene, whereas no change in methylation was observed in the proto-oncogene *c-myc* or *c-fos* (41). Methylation is thought to be involved in the activation (hypomethylation) and inactivation (hypermethylation) of promoter activity of cellular genes. Coming back to the issue of tumor prevention by

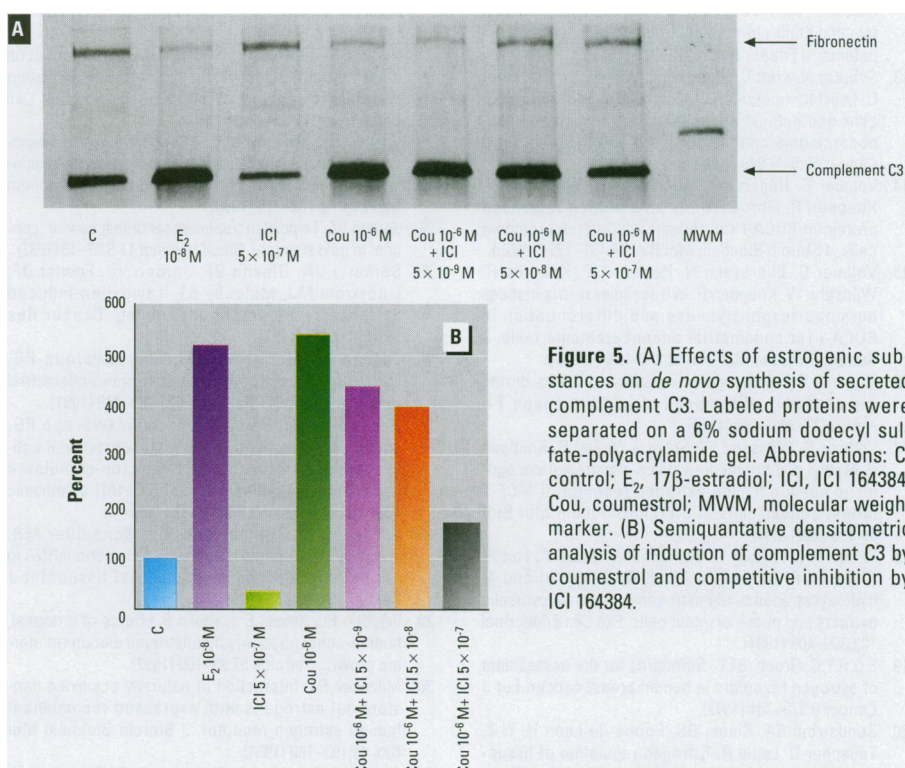


Figure 5. (A) Effects of estrogenic substances on *de novo* synthesis of secreted complement C3. Labeled proteins were separated on a 6% sodium dodecyl sulfate-polyacrylamide gel. Abbreviations: C, control; E₂, 17 β -estradiol; ICI, ICI 164384; Cou, coumestrol; MWM, molecular weight marker. (B) Semiquantitative densitometric analysis of induction of complement C3 by coumestrol and competitive inhibition by ICI 164384.

phytoestrogens, it is likely that phytoestrogens use different mechanisms to develop their cancer protective effects. Additionally, undiscovered properties of phytoestrogens such as tissue specificity might be responsible for other beneficial effects on human health regarding cardiovascular diseases or Alzheimer's disease.

In this study we were able to show that the extent of induction of molecular endpoints by the phytoestrogens coumestrol, genistein, and daidzein is not strongly correlated to the extent of their binding affinity to the ER. Furthermore, it could be proved that the regulation of increase of complement C3 is mediated by an increase of its mRNA level. The RUCA-I cell line represents a valuable endometrial-derived model to identify molecular mechanisms mediated by phytoestrogens.

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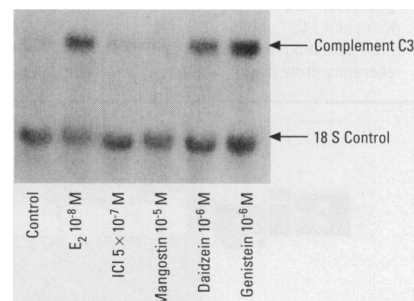


Figure 6. Effects of phytoestrogens mangostin, daidzein and genistein on steady state mRNA level of complement C3. Hybridization with 18S probe was used as control for the amount of RNA loaded.

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- An environmental genome study of how disease-susceptibility genes vary from person to person in a representative sector of the population, to help us see why you might get a nerve disorder from an exposure to a chemical whereas your friend Lee might not . . . information to protect you and Lee, without guesstimates and fudge factors.
- A survey of chemicals polluting our blood—a look at what we, as a population, have been exposed to.
- Customized mice to quickly screen chemicals and drugs, and help conquer diseases like breast cancer.
- A study of mixtures. We don't face chemicals one on one. Why should science?

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